

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07H 15/24	A1	(11) International Publication Number: WO 98/54195 (43) International Publication Date: 3 December 1998 (03.12.98)
(21) International Application Number: PCT/US98/10888 (22) International Filing Date: 28 May 1998 (28.05.98) (30) Priority Data: 08/866,706 30 May 1997 (30.05.97) US (71) Applicants: BION, INC. [US/US]; 5th floor, 840 Memorial Drive, Cambridge, MA 02139 (US). THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US). (72) Inventors: GALLAGHER, Rex, T.; 22 Old Town Road, Beverly, MA 01915 (US). HAUPERT, Gamer, T., Jr.; 512 Great Road, Littleton, MA 01460 (US). (74) Agents: ELMORE, Carolyn, S. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02173 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: LARGE-SCALE ISOLATION AND PURIFICATION OF HIF		
(57) Abstract The present invention relates to a method of isolating and/or purifying hypothalamic inhibitory factor (HIF) from a sample (e.g., tissue fluid) containing HIF. The present invention provides for isolation of large amounts of HIF using diafiltration, solid phase extraction and immunoaffinity techniques. In one embodiment, the invention relates to a method of purifying hypothalamic inhibitory factor from a sample containing hypothalamic inhibitory factor comprising subjecting the sample to diafiltration, solid phase extraction and immunoaffinity chromatography. In a particular embodiment, the invention relates to subjecting the sample to diafiltration to produce a diafiltrate of HIF; subjecting the diafiltrate to a first solid phase extraction (SPE) to produce a first fraction of HIF; subjecting the first fraction to immunoaffinity chromatography, wherein an antibody which binds to HIF is coupled to an immunoaffinity column, to produce a second fraction of HIF; subjecting the second fraction of HIF to reverse phase HPLC chromatography to produce a third fraction of HIF; and recovering purified HIF from the third fraction.		

A02

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AI.	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NR	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

-1-

LARGE-SCALE ISOLATION AND PURIFICATION OF HIF

BACKGROUND

Hypothalamic inhibitory factor (HIF), an isomer of ouabain, is a physiologic inhibitor of membrane-bound Na^+ , K^+ -activated adenosine triphosphate (Na^+ , K^+ -ATPase) and produces a positive inotropic effect on cardiac cells. Positive inotropic effect means that the contractility of the cardiac cells is enhanced in a dose-dependent manner. HIF can be administered to a mammalian host to treat cardiac malfunction (e.g., congestive heart failure, paroxysmal atrial tachycardia, atrial fibrillation and flutter). Use of HIF for treating cardiac malfunction is further described in U.S. patent application No. 08/338,264, filed November 10, 1994.

HIF has been purified to homogeneity using an affinity chromatography method in which purified canine renal Na^+ , K^+ -ATPase is coupled to paramagnetic particles through a glutaraldehyde bridge (Tymiak, A.A., et al. *Proc. Natl. Acad. Sci., USA*, 90:8189-8193 (1993)). The enzyme immobilizes bound HIF in the presence of Mg^{++} and inorganic phosphorous with high affinity, and after washing away contaminating materials, HIF is eluted from the affinity column by chelating Mg^{++} with EDTA. With a subsequent HPLC step, this technique produced enough pure HIF to allow structure determination and the differentiation of HIF from ouabain (Tymiak, A.A., et al. *Proc. Natl. Acad. Sci., USA*, 90:8189-8193 (1993)). Unfortunately, losses at the affinity step are substantial, with activity recovery averaging only 30%. Also, the method is laborious in the sense that pure enzyme needs to be repeatedly prepared from fresh canine renal medulla.

-2-

Thus, a need exists for an improved method of isolating and/or purifying HIF. In particular, there is a need for large-scale preparative methods for purifying HIF.

5 SUMMARY OF THE INVENTION

The present invention relates to a method of isolating and/or purifying hypothalamic inhibitory factor (HIF) from a sample (e.g., tissue fluid) containing HIF. The present invention provides for
10 isolation of large amounts of HIF using diafiltration, solid phase extraction and immunoaffinity techniques; preferably all three techniques are used. However, it should be recognized that each of the steps of the process could be used alone or in any combination. For
15 example, diafiltration, the solid phase extraction and the immunoaffinity technique described herein may be used alone or in combination with other known methods to purify and/or isolate HIF from an appropriately prepared sample, e.g., a sample chromatographically treated. In
20 one embodiment, the invention relates to a method of purifying hypothalamic inhibitory factor from a sample containing hypothalamic inhibitory factor comprising subjecting the sample to diafiltration, solid phase extraction and immunoaffintiy chromatography. In a
25 particular embodiment, the invention relates to subjecting the sample to diafiltration to produce a diafiltrate of HIF; subjecting the diafiltrate to a first solid phase extraction (SPE) to produce a first fraction of HIF; subjecting the first fraction to
30 immunoaffinity chromatography, wherein an antibody which binds to HIF is coupled to an immunoaffinity column, to produce a second fraction of HIF; subjecting the second fraction of HIF to a second SPE to produce a third fraction of HIF; and recovering purified HIF from the
35 third fraction.

-3-

In one embodiment, the present invention relates to a method of purifying HIF wherein a tissue slurry from a sample comprising HIF is prepared. A retentate of the tissue slurry, which includes HIF is then prepared. The
5 retentate is diafiltered to produce a diafiltrate which includes HIF which is chromatographically treated using solid phase extraction. Immunoaffinity chromatography is then performed on the HIF using an antibody (e.g., a monoclonal antibody) which binds to HIF. Following
10 immunoaffinity chromatography, reverse phase high pressure liquid chromatography (RPHPLC) can be performed to produce further purified HIF. An example of a monoclonal antibody which binds HIF is 26-10 (Mudgett-Hunter, M., et al., *J. Immunol.*, 129:1165-1172 (1982)).

15 In another embodiment, the invention relates to a method of purifying HIF wherein tangential diafiltration is performed on a sample comprising HIF to produce a diafiltrate permeate which includes the HIF. SPE is then performed on the diafiltrate permeate to produce a
20 concentrate of HIF. Following SPE, immunoaffinity chromatography is performed on the concentrate using an antibody having affinity for HIF to produce a HIF-containing fraction. RPHPLC, or a similar technique, performed on the HIF fraction follows which results in
25 the production of purified HIF.

The present invention also relates to a method of purifying HIF wherein a tissue comprising HIF is blended to form a tissue slurry, which is centrifuged to produce a retentate comprising the HIF. The retentate is
30 tangentially diafiltered to produce a diafiltrate permeate comprising the HIF. The diafiltrate permeate is chromatographically treated by SPE employing an acetonitrile elution and a silica packing to produce a first HIF fraction in acetonitrile. The first fraction
35 is concentrated and then chromatographically treated by immunoaffinity chromatography employing a glycine elution and a resin packing, wherein an antibody which

-4-

binds to HIF is coupled to the resin, to produce a fraction of HIF in glycine. The fraction of HIF in glycine is chromatographically treated by RPHPLC employing an acetonitrile gradient elution and a silica
5 packing to produce a second HIF fraction in acetonitrile. The acetonitrile is then evaporated from the second HIF fraction thereby producing purified HIF.

Purification of HIF using the methods described herein provides for at least a 15-fold increase in yield
10 of pure HIF over the prior art processes.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graph of log concentration (M) versus % inhibition showing the binding curves of ouabain and HIF to monoclonal antibody 26-10.

15 Figure 2 are the results of mass spectrometry for determination of the molecular weight of the purified HIF.

Figure 3 is a chromatograph of analytical high pressure liquid chromatography isolation of purified HIF
20 in which a C18 column and an acetonitrile:water gradient with UV detection at 220 nm was used.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of purifying HIF from a sample (e.g., tissue or fluid)
25 containing HIF. The isolation and purification scheme for HIF is based in part on the discovery that the following combination of key properties of HIF: that HIF is a small, low molecular weight molecule (MW 584) and highly water soluble, allows exploitation of purifying
30 HIF using any, and preferably all or a combination of the following diafiltration, solid phase extraction and immunoaffinity on an industrial scale. In a particular embodiment, tangential diafiltration technology, coupled in 'tandem' to solid phase extraction is used to provide
35 an efficient isolation procedure to remove the HIF from

-5-

the tissue or fluid source. Subsequent purification by immunoaffinity chromatography, optionally followed by reverse phase high performance liquid chromatography, yields purified HIF.

5 As defined herein, purified HIF refers to HIF which is substantially free of or isolated from other tissue or fluid protein components and contaminants. In addition, the purified HIF of the present invention has at least 40% activity recovery. Preferably, the
10 purified HIF has about 50-75% activity recovery, more preferably the purified HIF has about 75-90% activity recovery and most preferably, the purified HIF has about 90-95% activity recovery. As defined herein, "activity recovery" of HIF is the activity of HIF as measured
15 indirectly by, for example a biological assay such as specific inhibition of $\text{Na}^+\text{-K}^+\text{-ATPase}$ pump activity in human erythrocytes, or as measured directly, for example, by UV absorbance following HPLC chromatography.

HIF can be purified from any mammalian (e.g.,
20 bovine, murine, human) sample, such as a tissue or fluid source, in which HIF is present using the methods described herein. For example, as demonstrated herein, HIF can be isolated from bovine hypothalamus. Other suitable samples which can be used in the methods of the
25 present invention include, but are not limited to, blood, blood plasma, urine, muscle, placental tissue, lymph, cerebrospinal fluid, cardiac tissue, adrenal tissue, pituitary glands, kidney, and/or renal tissue. It is understood that the HIF source can be from a
30 freshly collected tissue or fluid sample or from an old sample previously maintained, for example, in a frozen state.

In the embodiment in which the sample is blood, at or soon after collection, the blood can be mixed with at
35 least one anticoagulant to prevent significant clotting of the blood. Suitable anticoagulants for blood are as classically known in the art and include, for example,

-6-

sodium citrate, ethylenediaminetetraacetic acid and heparin. When mixed with blood, the anticoagulant may be in a solid form, such as a powder, or in an aqueous solution.

5 As described in the exemplification, in one embodiment of the present invention a slurry is prepared from the tissue or fluid source from which the HIF is being purified. Suitable methods for preparing a slurry include blending the tissue or fluid with a suitable
10 water miscible solvent (e.g., alcohol, methanol, ethanol, glycol, dimethylformamide, acetone, dimethylsulfoxide, acetonitrile and other such solvents). It is understood that other methods generally known in the art for preparing a slurry from a
15 tissue or fluid sample can be employed. For example, a homogenizer, such as a Waring blender can be used.

Following preparation of the slurry, a retentate of the slurry which includes HIF is prepared by decanting a supernatant. Centrifugation of the slurry can be used
20 to obtain a retentate which includes HIF. Optionally, the slurry can be centrifuged at, for example, about 4000 G for about 25 minutes. It is preferred that the retentate obtained from the tissue slurry contains a minimum concentration of about 500 units bioactivity HIF
25 which is approximately 219 ng HIF per kg starting tissue. It is understood that other methods generally known in the art for preparing a retentate from a slurry can be employed to obtain HIF in the retentate. For example, filtration can also be used to obtain a
30 retentate which includes HIF.

Ultrafiltration can then be used on the retentate which includes HIF to remove large and/or high molecular weight components. In ultrafiltration procedures on biological extracts, concern arises from the potential
35 of proteins that might bind generally or specifically to the small amounts of desired solute molecules, such as HIF molecules, in the very complex soup of biomolecules

-7-

that constitutes the extract. Literature descriptions of purification procedures for endogenous digitalis-like factor (EDLF) and ouabain-like compounds (OLC) suggest that such a mechanism could well operate in the case of
5 ultrafiltration purification procedures for HIF from slurry preparations (Valdes, R. Jr., et al., *J. Clin. Endocrinol. Metab.*, 60:1135-1143 (1985)). In a preferred embodiment, continuous diafiltration can be used to circumvent the problem associated with
10 ultrafiltration, both for solutions and for particulate-laden preparations.

Diafiltration is performed on tissue or fluid samples in which HIF is present to remove larger or high molecular weight tissue components such as proteins,
15 polysaccharides, peptides, lipoproteins and nucleic acids, with a molecular weight above about 3000 daltons. Acceptable diafilters for use in the present invention include, for example, filters made by Pall (e.g., Pall Filtron, catalogue number OS003G05, Omega 3 KD Open
20 Channel 30 mil 4 ft² cassette), Millipore and A/G Technology. Preferred characteristics of the diafilters for use in the present invention include narrow pore-size distribution, high flow rates, stability against biological degradation, stability against organic
25 solvents (e.g., methanol, ethanol), good permeability performance and/or good membrane recovery after cleaning. In a particular embodiment, the diafilter is such that protein binding to the surface and interstitial structure of the membrane filter occurs to
30 a minimum (less surface fouling). Preferred diafilter polymers include, for example, polyether sulfone (e.g., a modified Pall polyether sulfone membrane), cellulose and/or regenerated cellulose filters. The diafiltration step can be performed using numerous techniques.

35 Diafiltration is the fractionation process of "washing" smaller molecules through an ultrafiltration membrane, leaving larger molecules in the retentate.

-8-

The process of continuous diafiltration is achieved by adding solvent to the retentate at the same rate as solvent and small molecules pass into the filtrate. The diafiltration volume is the volume equal to the initial process sample or feed volume in which the molecule of interest occurs. The number of diafiltration volumes required for a successful fractionation depends on several factors, most particularly on how readily the molecule of interest is retained or permeates through the membrane. For molecules that have no membrane retention (retention=0%, i.e. fully permeates the membrane) such as salts and solvents, 7 diafiltration volumes are required for 99.9% removal from the retentate. For a molecule that is retained 25%, 9 volume exchanges are required (Membrane Separation Products catalog, (1995/1996) Filtron Corporation, Northborough, MA 01532, see pages 9, 10, & Table 2). For purification of HIF using the methods described herein, the number of diafiltration volumes (DVs) used can range from about 5 DVs to about 30 DVs. In a preferred embodiment, the number of DVs used can range from about 7 DVs to about 20 DVs, and most preferred can range from about 15 DVs to about 20 DVs.

Solvents for use in diafiltration can be any solvent which is compatible with the aqueous solvent system used in the diafiltration process and include, for example, deionized water (DIH_2O), sodium chloride, sodium acetate and phosphate buffers (e.g., Tris).

In one embodiment, the diafiltration is performed using a continuous diafiltration method. In a preferred embodiment, tangential diafiltration is used. Tangential diafiltration produces a "sweeping" action across the membrane surface; this sweeping action keeps the retained macromolecules in the retentate phase from accumulating at the membrane surface, thus minimizing concentration polarization and membrane fouling. Thus, in tangential dialysis, sample is recirculated across

-9-

the top of the membrane - "tangentially" to the membrane surface - while applied pressure causes solute and small molecules to pass through the membrane.

For example, hollow fiber, spiral membrane cartridges, and open-channel membrane cartridges, which are based on tangential flow technology, can be used. In a preferred embodiment, tangential diafiltration is performed using a linear-open-channel separator. As described in the exemplification, the Pall Filtron Corporation technology of membrane "cassettes" with retentate channels processing linear-open-channel separators can be used (Membrane Separation Products catalog, (1995/1996) Filtron Corporation, Northborough, MA 01532, see pages 9, 10, & Table 2). The cassette technology consists of multiple layers of ultrafiltration (UF) membrane assemblies composed of sheets of UF membrane placed alternately between filtrate screen separators, and retentate screen separators, in a sandwich-like arrangement. Blocked borders on the filtrate and retentate screens direct the separated fractions to collection ports on the manifold. These cassettes allow cells and particulate matter to move freely above the membrane surface at low shear conditions without plugging. A key concept of the cassette technology is that cassettes can be stacked in multiple units, enabling large batch volumes of feed solutions and broths to be processed. Scale-up simply requires increased membrane area, obtained by adding more cassettes, and increased pump capacity. Optionally, one or more ultrafiltration steps can be performed after diafiltration or in between diafiltration steps. For example, diafiltration is performed on the supernatant as described herein. Then ultrafiltration (addition of buffer is stopped) is performed on the retentate until the retentate volume is reduced. Thereafter, diafiltration can again be commenced until the desired DV is achieved.

-10-

Following diafiltration of retentate of the sample (e.g, tissue) slurry, solid phase extraction (SPE) can be performed on the diafiltrate of HIF. It should be recognized that it may be desirable to perform SPE before and/or after diafiltration although diafiltration is preferably performed first. The solid phase extraction can be performed using numerous techniques. For example, the diafiltrate of HIF is directed into one or more SPE chromatographic columns or cartridges to further separate the HIF from other contaminants such as ions, fatty acids, phospholipids, amino acids, sugars and/or steroids. Examples of suitable media or packing of the chromatographic columns or cartridges include but not limited to reverse phase silicas (e.g., C4, C8, C12, C18), polyvinylpyrrolidone (e.g., HP-20), various cellulose derivatives (e.g., acetate cellulose) and cyclodextrins (e.g., α , β , γ and derivatives thereof). In a preferred embodiment, SPE chromatographic columns or cartridges contain a silica based packing suitable to separate HIF from additional contaminants. Suitable silica based column packings include, for example, C4, C8, C12 and C18 columns.

Chromatographic columns are first pre-treated by flushing with a first eluant which facilitates HIF binding. Diafiltered HIF is then injected onto the media columns. After injecting the diafiltered HIF, the chromatographic columns are then successively washed with different eluants to produce an HIF fraction which has been further purified.

An acetonitrile/water eluant is used to remove protein etc. on the SPE chromatographic column or cartridge. In a particular embodiment, a C18 SPE technique is used to extract HIF from the large volume of aqueous permeate generated in the tangential diafiltration of the bovine hypothalamic supernatant. The SPE step captures the small amount of the desired HIF molecule in the dilute aqueous solution/slurry;

-11-

elution with about 30-60% aqueous acetonitrile then yields the HIF in a highly concentrated SPE fraction.

Following SPE, the HIF fraction can be further concentrated under vacuum, for example, on a Buchi
5 rotary evaporator and/or a Savant SpeedVac vacuum concentrator. It is understood that other methods generally known in the art for concentrating purified fractions obtained from SPE can be employed.

Affinity chromatography can then be performed on
10 the highly concentrated SPE fraction. In one embodiment, affinity chromatography can be performed using the Na⁺-K⁺-ATPase inhibitor. In a preferred embodiment, immunoaffinity chromatography is performed on the HIF in a highly concentrated SPE fraction or on
15 the HIF fraction which has been further concentrated under vacuum. In another embodiment, an HIF containing fluid or tissue sample which is free of high molecular weight tissue components is subjected to immunoaffinity chromatography. Concentrated HIF is directed onto one
20 or more chromatographic immunoaffinity columns to produce an immunoaffinity column eluate which includes HIF further separated from other contaminants such as steroids (e.g., corticosteroids), fatty acids and other small organic molecules. Examples of suitable media or
25 packing of the chromatographic immunoaffinity columns include various sepharose resins to which is bound an antibody which binds to HIF. In a preferred embodiment, chromatographic immunoaffinity columns contain a sepharose, polydextran carbohydrate, D-galactose, 3-6
30 anhydrol galactose resin or packing which can be coupled to an antibody which binds HIF facilitating further separation of HIF from additional contaminants. Suitable chromatography immunoaffinity column packings include cyanogen bromide activated Sepharose 4B resin,
35 Sephadex 300 and Sephacryl 300.

Antibodies for use in the methods of the present invention include antibodies which bind to HIF (anti-HIF

-12-

antibodies). In one embodiment, the antibody specifically recognizes HIF. In another embodiment, the antibody reversibly binds to the HIF. In a preferred embodiment, the antibody has good affinity for HIF (e.g., the affinity of monoclonal antibody 26-10) and low cross reactivity with other mammalian steroids. The binding affinity of the antibody to HIF for use in the present invention is from about 3×10^{-7} M to about 5×10^{-7} M. In one embodiment, the anti-HIF antibody has a binding affinity of about 3×10^{-7} M for HIF. In a preferred embodiment the antibody for use in the methods of the present invention is a monoclonal antibody. In a particular embodiment, the monoclonal antibody is 26-10 (Mudgett-Hunter, M., et al., *J. Immunol.*, 129:1165-1172 (1982)). Antibody fragments (e.g., Fab fragments; an HIF antibody binding fragment) can be used in the methods of the present invention. For example, an HIF binding fragment of mAb 26-10 can be used.

An antibody which binds HIF can be coupled to the immunoaffinity column packing using numerous techniques. For example, the antibody is contacted with the packing or solid phase matrix of the immunoaffinity column and a chemical molecule which crosslinks the antibody to the packing. Methods for coupling antibodies (e.g., covalently) to solid phase matrices include, for example, use of A/G beads, activated beads and/or activated antibodies. (See e.g., *Antibodies, A Laboratory Manual*, by Ed Harlowe and David Lane (1988), which is herein incorporated by reference).

Elution of HIF from the immunoaffinity column can be accomplished using a variety of techniques. For example, the immunoaffinity column to which is bound HIF can be treated with harsh conditions, a saturating amount of a compound which mimics the binding site and/or an agent that induces allosteric changes resulting in release of HIF. In one embodiment, a glycine elution is used to remove the HIF from the

-13-

immunoaffinity column. (See e.g., *Antibodies, A Laboratory Manual*, by Ed Harlowe and David Lane (1988), Chapter 13).

In a preferred embodiment, aqueous alcohol (e.g., methanol) is used to elute HIF from the immunoaffinity column. Many proteins and enzymes are easily denatured by a variety of physio-chemical factors. Loss of "active" conformational states of such biomolecules, by unfolding of the molecules, and subsequent refolding to other (unnatural) conformations, or even complete loss of ability to refold, usually means a loss of key properties; if the ability to return to the useful conformation(s) is permanently lost, then the molecules are 'denatured' and are no longer of use. Exposure to organic solvents, including significant concentrations of alcohols, such as methanol and ethanol, is often enough to permanently denature many enzymes and proteins. However, as shown herein, in the case of affinity media prepared by immobilization of an antibody onto a stable supporting matrix, antibody stability can be achieved to moderate levels of alcohol (e.g., methanol, ethanol) concentrations in aqueous solutions.

After immunoaffinity chromatography, reverse phase high pressure liquid chromatography (RPHPLC) can be, and is preferably, performed. However, a variety of techniques can be used in place of or in addition to RPHPLC. Today, RPHPLC is the most common form of HPLC. In RPHPLC, water-containing mobile phases are used with column packings that have a hydrophobic surface layer. The immunoaffinity eluate of HIF can be directed, for example, onto one or more HPLC columns to further separate HIF. Suitable HPLC column packings include reverse phase silicas (e.g., C4, C8, C12, C18), cyclodextrins (e.g., α , β , γ and derivatives thereof), phenyl, CN and NH₂ packings. In a preferred embodiment, end-capped silica is used. Chromatographic columns are first pre-treated by flushing with a first column eluant

-14-

which facilitates HIF binding. After injecting the immunoaffinity eluate of HIF onto the column, the chromatographic columns are then successively washed with different eluants and/ or gradients to produce an
5 HIF fraction which has been further purified.

For example, an acetonitrile gradient can then be used to remove the HIF from the RPHPLC column. In a particular embodiment, an analytical C18 HPLC column can be used as a "preparative" column at the final stage of
10 purification. Since only μg of substance are being injected in a run, the full resolution capabilities of the column can be realized. Fractions collected from an "analytical" run, yield pure HIF, which can be determined using, for example, a characteristic UV
15 spectrum, a bioassay and/or electrospray mass spectroscopy (ESMS). Repeat runs, and columns additional to C18, can also be used to give HIF of the highest purity.

The purification procedure described herein has
20 several advantages over previous methods. For example, final yields of pure HIF are increased at least 15-fold. The antibody column is stable and can be reused at least ten times without loss of activity. Scale-up to accommodate larger front-end preps is easily
25 accomplished since 26-10 can be produced in whatever quantities are needed as described below. Purified HIF of the present invention can be used to treat cardiac malfunction as further described in U.S. patent application No. 08/338,264, filed November 10, 1994,
30 which is incorporated by reference.

This invention is illustrated further by the following exemplification which is not to be construed as limiting in any way.

-15-

EXEMPLIFICATION

EXAMPLE 1 PURIFICATION OF HIF

Tissue is processed in a batch-mode operation, 1 Kg tissue per batch. Scale-up operations can be performed, either with additional processing lines of equipment, or with larger industrial-scale systems, readily available from the equipment suppliers.

Tissue slurry preparation:

The tissue sample of frozen bovine hypothalamus, or other animal tissue or fluid, 1Kg, was placed in a high-speed blender (e.g. Waring, one gallon 'Commercial Heavy Duty Blender'), with 1.5L MeOH, and blended for 3x20 seconds low, 3x20 seconds on medium and 2x20 seconds on high speed. The resulting slurry was decanted from the blender into a glass jar bath (e.g. Kimax, 7.5L, 83/4" x 10"), and the blender was then rinsed out successively with 1.0L, then 1.5L MeOH, the rinsing being added to the glass jar bath. The slurry was left to stir overnight at 300rpm at 4°C.

20 *Supernatant preparation:*

The stirred slurry was decanted into centrifuge bottles (e.g. 6 x 1L, Nalgene, with screw caps), and centrifuged at 4,000 G for 25 min at 12°C (e.g. Marathon 12KBR centrifuge). The resulting supernatant phase was poured into a glass jar bath (e.g. Kimax, as above). This supernatant phase is referred to as the "retentate".

Diafiltration:

Diafiltration was then performed on the supernatant. In one procedure, the retentate was pumped through a tangential ultrafiltration membrane system (e.g. Pall Filtron polyethersulfone (PES) membranes, Omega Cassette 3kD Open Channel, 6-cassette stack, in a

-16-

Maximate-Ext stainless steel cassette holder (or equivalent equipment), total membrane surface area = 24ft²), with the pump speed and control valves adjusted for an average retentate flux of 5.5LMH, and a retentate flow rate = 200 - 220 ml/min; diafiltration "buffer" (= DIH₂O, in this application), 50L, was fed via a peristaltic pump into the retentate jar bath, at a flow rate = permeate flow rate, so that constant volume in the retentate jar bath was maintained.

Typically, for an average filtrate flux = 217ml/min, a run takes 3.8 - 4.0 hours to generate 50L filtrate (permeate). Collection of 50L permeate gives a diafiltrate volume (DV) value of ca. 10. Transmembrane pressures required in this process are typically 3 - 4 psi.

The diafiltration cassette stacks and equipment were cleaned with NaOH (0.5N, 50°C), NaOCl (400ppm), and PBS buffer (0.1M, pH 7.0), followed by a H₂O wash, to return them to their original condition, prior to the next batch being processed.

In a variation of the above procedure, diafiltration was performed as follows: 1L DIH₂O was added to the initial retentate slurry in the jar bath, to give an initial bath volume of ca. 5.0L, and diafiltration was performed as above, for ca. 0.5 - 1.5hr, until 5-10L permeate was collected. Then, ultrafiltration was performed (i.e. addition of H₂O to the retentate jar, via the peristaltic pump, was stopped) on the retentate, until the retentate volume was reduced to 4L. Thereafter, diafiltration was again commenced, and continued until the total permeate volume collected was 50L.

This procedure has the advantage of exposing the membranes in the cassette stacks to a lower initial concentration of MeOH, which helps reduce a tendency for the membranes to swell and give low transmembrane flow rates, with high transmembrane pressures (TMP's).

-17-

*Solid Phase Extraction of HIF from Diafiltration**Permeate:*

Accumulated permeate from above, containing HIF, was pumped through a C18 SPE column (Biotage Flash 75M cartridge, 7.5 x 15cm, custom-packed with end-capped, high-carbon load (ca. 18-22%) C18 silica), at a flow rate of 220-280ml/min, retaining the HIF on the column. The column was then washed with 4L H₂O, then the HIF was eluted with 2L 80% aqu ACN. Collected fractions were bioassayed to locate HIF, and HIF-containing fractions pooled and concentrated under vacuum on a Buchi rotary evaporator, and/or a Savant SpeedVac vacuum concentrator.

*Purification via Affinity Chromatography with Digoxin**Antibodies:*

As described below, affinity purification of the HIF in the concentrate was achieved with anti-digoxin monoclonal antibody immobilized on Sepharose. The fractionation was guided by bioassay, and HPLC with UV detection.

Studies indicated that some monoclonal anti-digoxin antibodies recognized ouabain (Panka, D.J. and Margolies, M.N., *J. of Immunol.*, 132:2385-2391 (1987)). Several available anti-digoxin antibodies were studied to determine relative affinity for ouabain. In an ¹²⁵I competition plate assay using goat-anti-mouse IgG Fab to bind the antibodies, it was found that one of the antibodies, 26-10 (Mudgett-Hunter M. et al., *J. Immunol.* 129:1165-1172, (1982)) bound ouabain with a relative affinity (IC₅₀) of 3 x 10⁻⁷ M. 26-10 was then studied for binding of ouabain and HIF in side by side experiments using biologically equivalent amounts of the two inhibitors based on the standard human erythrocyte ⁸⁶Rb⁺ uptake assay. Figure 1 shows that ouabain and HIF binding curves were virtually superimposable.

-18-

Monoclonal antidigoxin antibody 26-10 was obtained from A/J mice immunized with a digoxin-human serum albumin (Dig-HSA) conjugate and spleen cells were fused with the non-secreting murine myeloma Sp2/0 cell line (Mudgett-Hunter, M., et al. *J. Immunol.*, 129:1165-1172 (1982)). Hybridoma cells identified by radioimmunoassay (RIA) as secreting anti-digoxin antibody were cloned and grown in tissue culture and amplified in the ascites form in mice. Digoxin specific hybridoma proteins were purified from ascites by a variety of chromatographic methods, including ion-exchange chromatography on DEAE cellulose and affinity chromatography on Digoxin-BSA-Sepharose or ouabain-amine Sepharose.

Coupling of Antibody 26-10 to Sepharose-4B

Antidigoxin antibody 26-10 was coupled to cyanogen bromide activated Sepharose ("Affinity Chromatography, Principles and Methods", Pharmacia LKB Biotechnology Series). CNBr activated Sepharose 4B resin was suspended in 1 mM HCl solution. The swelled gel was washed with the same HCl solution on a sintered glass filter. After the HCl solution was sucked off and cracks appeared in the gel, the gel was then immediately transferred to the solution of antibody 26-10. The antibody solution was previously prepared by dialyzing against 0.1 M NaHCO₃ buffer (pH 8.3) containing 0.5 M NaCl. The coupling reaction was carried out at 4°C with gentle shaking for 3 days; the immobilization yield was 97%. The Ab-Sepharose resin is stored in 0.1 M phosphate buffered saline (pH 7.2) containing 0.1% sodium azide (PBSA).

Elution Conditions of Affinity Column and Reuse

In pilot studies using ³H-ouabain, various eluants were studied to determine an optimal recovery procedure. 0.2 M glycine (pH 2.5) provides consistently efficient elution of ouabain and HIF (latter determined by

-19-

bioassay units of activity before and after the chromatography). If washed and stored in PBSA at 4°C between use, the affinity resin complex shows good stability. No obvious decrease in binding efficiency has been noted in over 10 repeated uses in the purification of HIF.

Affinity-HPLC Separation of HIF

Dried HIF from the Biotage SPE column was dissolved in 0.1 M PBS buffer (pH 7.2) and loaded onto a 10 ml Ab-resin column and incubated at 4°C overnight. The supernatant is allowed to pass through the column and the resin was washed with PBS (15 ml x 3). The affinity column was then eluted with 0.2 M glycine solution (pH 2.5, 10 ml x 5).

The combined glycine elution solution was filtered and loaded onto a semi-preparative mBondapak C18 column (Waters, 10 mm, 125Å, 19 mm x 150 mm). The column was washed (6 ml/min) with deionized water eluting the glycine (UV detection at 220 nm), with continued water wash for an additional 30-40 min, followed by elution with a gradient of 0-30% acetonitrile at a flow rate of 6 ml/min. HIF elutes at 16-18 min into the gradient as detected by bioassay.

Alternatively, 1 ml columns of the affinity medium, packed in 0.1 M PBS/0.1% Na azide, were first washed free of azide with 5 ml PBS at a flow rate of 1 drop/sec. Then 1 ml of HIF solution (dissolved in 0.1 M PBS) was loaded on the column and allowed to equilibrate for 40 minutes. The column was washed with 5 ml 0.1 M PBS and 5 ml DI H₂O. HIF was eluted from the column with 10 ml 40% aqueous MeOH (40 MeOH:60 H₂O) and fractions of the eluate were collected. The column was equilibrated with 5 ml 0.1 M PBS/0.1% Na azide, and stored in the cold until needed again.

-20-

Final Purification via HPLC on Analytical C18 Columns

The pooled, HIF-containing fraction was concentrated under vacuum (Buchi evaporator and/or Savant SpeedVac concentrator), and applied to an analytical C18 column, with aqueous ACN gradients at a flow rate of 1.0 ml/min. The procedure utilized a gradient program as follows: from 95% H₂O:5% ACN to 70% H₂O:30% ACN over 20 min, then to 10% H₂O:90% ACN over 5 min, hold for 1 min, then back to 95% H₂O:5% ACN over 5 min. Under these conditions, HIF has a retention time of ca. 14.2 min, identical to that of ouabain, on an Alltech Alltima C18 column, 4.6x250mm, 3 μ silica particle size. Fractions were collected and bioassayed (see above), and also monitored by UV diode-array detection, and LC/MS if desired. HIF-pure fractions are pooled and evaporated to give pure HIF.

RESULTS

The middling affinity of mAb 26-10 for HIF is desirable since it binds HIF, but not so avidly that elution is problematic. A method to purify HIF using mAb 26-10 coupled to cyanogen bromide activated Sepharose 4B was devised. Binding was done in phosphate buffered saline (PBS) and elution with 0.2 M glycine, pH 2.5. The eluate was chromatographed by semipreparative and then analytical C18 HPLC. Bioactivity eluted in a narrow peak (see Figure 3), and was characterized for yield in the erythrocyte ⁸⁶Rb⁺ uptake assay. Table 1 shows the results of three representative experiments. As can be seen, there is a substantial increase in specific bioactivity following the 26-10 affinity purification step.

-21-

Table 1: Activity Yields of HIF Pre and Post-Affinity
Purification using Monoclonal Antidigoxin Ab
26-10

Purification Steps	Prep. 2.6 (2 Kg tissue)	Prep. 2.7 (2 Kg tissue)	Prep. Pool 2 (1.4 Kg tissue)
Pre-Affinity CHP20 Column	200 units (100 units/Kg tissue)	500 units (250 units/Kg tissue)	1200 units (857 units/Kg tissue)
Post-Affinity Semiprep. HPLC	16,000 units (8000 units/Kg tissue)	10,000 units (5000 units/Kg tissue)	10,000 units (7143 units/Kg tissue)
Anal. HPLC	14,000 units (7000 units/Kg tissue)	8000 units (7000 units/Kg tissue)	9000 units (6429 units/Kg tissue)

Since the molecular weights of HIF and ouabain are identical (Tymiak et al., 1993), and since the affinity constants for binding to purified Na, K-ATPase are approximately the same for both inhibitors (~1 nM) (Hauptert, G.T., Jr., Carilli, C.T., and Cantley, L.C., *Am. J. Physiol.* 247:F919-F924, (1984), units of inhibition (1 U = 50% inhibition under standard conditions) can be converted into picomoles of ouabain-equivalent activity. In the RBC $^{86}\text{Rb}^+$ uptake assay, 1 U is approximately 0.75 pmol of ouabain or HIF. If each kg of starting hypothalamic tissue yields ~7000 units (Table 1), the following calculation can be made:

$$7000 \text{ U} \times 0.75 \text{ pmol/U} \times 584 \text{ pg/pmol} \div 10^6 = 3 \text{ } \mu\text{g HIF}$$

This yield is substantially greater than that afforded by our earlier methodology where no more than 1 μg HIF could

-22-

be expected per 5 kg starting tissue (Tymiak et al., 1993), and many preps were significantly below even that.

This means that, using the 26-10 affinity purification coupled with semipreparative HPLC, as much
5 as 15 μ g HIF per 5 kg prep can be reproducibly purified, and used to further characterize HIF.

EXAMPLE 2 MASS SPECTROMETRY OF PURIFIED HIF

To verify that the inhibitor purified using an anti-digoxin antibody was that isolated by the enzyme affinity
10 technique (i.e., HIF), the molecular weight of the 26-10 purified material was determined by mass spectrometry. Figure 2 shows a mass of 585 (M + H) by FAB, and 607 (M + Na⁺) by MALDI-TOF, confirming the purified compound to be HIF (Tymiak, A.A., et al. *Proc. Natl. Acad. Sci., USA*,
15 90:8189-8193 (1993)).

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the
20 invention described herein. These and all other such equivalents are intended to be encompassed by the following claims.

-23-

CLAIMS

We claim:

1. A method of purifying hypothalamic inhibitory factor from a sample containing hypothalamic inhibitory factor comprising subjecting the sample to diafiltration, solid phase extraction and immunoaffinity chromatography.
2. The method of Claim 1 wherein the diafiltration is in tandem to the solid phase extraction.
3. The method of Claim 2 wherein the immunoaffinity chromatography follows the solid phase extraction.
4. A method of purifying hypothalamic inhibitory factor from a sample containing hypothalamic inhibitory factor comprising the steps of:
 - a) subjecting the sample to diafiltration to produce a diafiltrate of hypothalamic inhibitory factor;
 - b) subjecting the diafiltrate of a) to solid phase extraction to produce a first fraction of hypothalamic inhibitory factor;
 - c) subjecting the first fraction of b) to immunoaffinity chromatography, wherein an antibody which binds to hypothalamic inhibitory factor or an HIF binding fragment thereof is coupled to an immunoaffinity column, to produce a second fraction of hypothalamic inhibitory factor;
 - d) subjecting the second fraction of c) to reverse phase high pressure liquid chromatography to produce a third fraction of hypothalamic inhibitory factor; and
 - e) recovering purified hypothalamic inhibitory factor from the third fraction of d).

-24-

5. The method of Claim 4 wherein the diafiltration is continuous diafiltration.
6. The method of Claim 5 wherein the continuous diafiltration is a tangential diafiltration.
- 5 7. The method of Claim 4 wherein the solid phase extraction is performed using a C18 silica column and an acetonitrile elution.
8. The method of Claim 4 wherein the sample is homogenized hypothalamus.
- 10 9. The method of Claim 4 wherein the antibody is a monoclonal antibody having an affinity of 3×10^{-7} for hypothalamic inhibitory factor.
10. The method of Claim 9 wherein the monoclonal antibody is 26-10 or a hypothalamic inhibitory factor antibody binding fragment thereof.
15
11. The method of Claim 4 wherein the reverse phase high pressure liquid chromatography is performed by use of a C18 silica column and an acetonitrile gradient elution.
- 20 12. The method of Claim 1 wherein the immunoaffinity chromatography of c) is performed using a methanol gradient.
13. A method of purifying hypothalamic inhibitory factor comprising the steps of:
25
 - a) preparing a tissue slurry from a sample comprising hypothalamic inhibitory factor;
 - b) obtaining a retentate of the sample of a), wherein the retentate comprises hypothalamic inhibitory factor;

-25-

- c) diafiltering the retentate of b) to produce a diafiltrate of hypothalamic inhibitory factor;
d) chromatographically treating the ultrafiltrate of c) using solid phase extraction;
5 e) performing immunoaffinity chromatography on the hypothalamic inhibitory factor obtained in d) using an antibody which binds to hypothalamic inhibitory factor or a hypothalamic inhibitory factor antibody
10 binding fragment thereof;
f) performing reverse phase high pressure liquid chromatography on the hypothalamic inhibitory factor obtained in e);
thereby producing purified hypothalamic inhibitory
15 factor.
14. The method of Claim 13 wherein the diafiltration is continuous diafiltration.
15. The method of Claim 14 wherein the continuous diafiltration is a tangential diafiltration.
- 20 16. The method of Claim 13 wherein the solid phase extraction of d) is performed using a C18 silica column and an acetonitrile elution.
17. The method of Claim 13 wherein the sample is homogenized hypothalamus.
- 25 18. The method of Claim 13 wherein the antibody is a monoclonal antibody having an affinity of 3×10^{-7} for hypothalamic inhibitory factor.
19. The method of Claim 18 wherein the monoclonal antibody is 26-10 or a hypothalamic inhibitory
30 factor antibody binding fragment thereof.

-26-

20. The method of Claim 13 wherein the reverse phase high pressure liquid chromatography of f) is performed by use of a C18 silica column and an acetonitrile gradient elution.
- 5 21. A method of purifying hypothalamic inhibitory factor comprising the steps of:
- 10 a) performing tangential diafiltration on a sample comprising hypothalamic inhibitory factor to produce a diafiltrate permeate which includes the hypothalamic inhibitory factor;
- b) performing solid phase extraction on the diafiltrate permeate of a) to produce a concentrate of hypothalamic inhibitory factor;
- 15 c) performing immunoaffinity chromatography on the concentrate of b) using an antibody having affinity for hypothalamic inhibitory factor or an hypothalamic inhibitory factor antibody binding fragment thereof to produce a hypothalamic inhibitory factor-containing fraction; and
- 20 d) performing reverse phase high pressure liquid chromatography on the hypothalamic inhibitory factor-containing fraction of c) to produce purified hypothalamic inhibitory factor.
- 25 22. The method of Claim 21 wherein the diafiltration is continuous diafiltration.
23. The method of Claim 22 wherein the continuous diafiltration is a tangential diafiltration.
24. The method of Claim 21 wherein the solid phase extraction of b) is performed using a C18 silica column and an acetonitrile elution.
- 30

-27-

25. The method of Claim 21 wherein the sample is homogenized hypothalamus.
26. The method of Claim 21 wherein the antibody is a monoclonal antibody having an affinity of 3×10^{-7} for hypothalamic inhibitory factor.
27. The method of Claim 26 wherein the monoclonal antibody is 26-10 or a hypothalamic inhibitory factor antibody binding fragment thereof.
28. The method of Claim 21 wherein the reverse phase high pressure liquid chromatography of d) is performed by use of a C18 silica column and an acetonitrile gradient elution.
29. A method of purifying hypothalamic inhibitory factor comprising the steps of:
- a) blending a tissue comprising hypothalamic inhibitory factor to form a tissue slurry;
 - b) centrifuging the tissue slurry of a) to produce a retentate comprising the hypothalamic inhibitory factor;
 - c) tangentially diafiltering the retentate of b) to produce a diafiltrate permeate comprising the hypothalamic inhibitory factor;
 - d) chromatographically treating the diafiltrate permeate of c) by solid phase extraction employing an acetonitrile elution and a silica packing to produce a first hypothalamic inhibitory factor fraction in acetonitrile;
 - e) concentrating the first hypothalamic inhibitory factor fraction of d);
 - f) chromatographically treating the concentrated hypothalamic inhibitory factor of e) by immunoaffinity chromatography employing a glycine elution and a resin packing, wherein

-28-

an antibody which binds to hypothalamic inhibitory factor or a hypothalamic inhibitory factor antibody binding fragment thereof is coupled to the resin, to produce a fraction of hypothalamic inhibitory factor in glycine;

5 g) chromatographically treating the hypothalamic inhibitory factor of f) by reverse phase high pressure liquid chromatography employing an acetonitrile gradient elution and a silica

10 packing to produce a second hypothalamic inhibitory factor fraction in acetonitrile;

h) evaporating the acetonitrile from the second hypothalamic inhibitory factor fraction in acetonitrile of g) thereby producing purified

15 hypothalamic inhibitory factor.

30. The method of Claim 29 wherein the diafiltration is continuous diafiltration.

31. The method of Claim 30 wherein the continuous diafiltration is a tangential diafiltration.

20 32. The method of Claim 29 wherein the solid phase extraction of d) is performed using a C18 silica column and an acetonitrile elution.

33. The method of Claim 29 wherein the sample is homogenized hypothalamus.

25 34. The method of Claim 29 wherein the antibody is a monoclonal antibody having an affinity of 3×10^{-7} for hypothalamic inhibitory factor.

30 35. The method of Claim 32 wherein the monoclonal antibody is 26-10 or a hypothalamic inhibitory factor antibody binding fragment thereof.

-29-

36. The method of Claim 29 wherein the reverse phase high pressure liquid chromatography of g) is performed by use of a C18 silica column and an acetonitrile gradient elution.
- 5 37. A method of purifying hypothalamic inhibitory factor from a sample containing hypothalamic inhibitory factor comprising subjecting the sample to diafiltration and immunoaffinity chromatography.

1/3

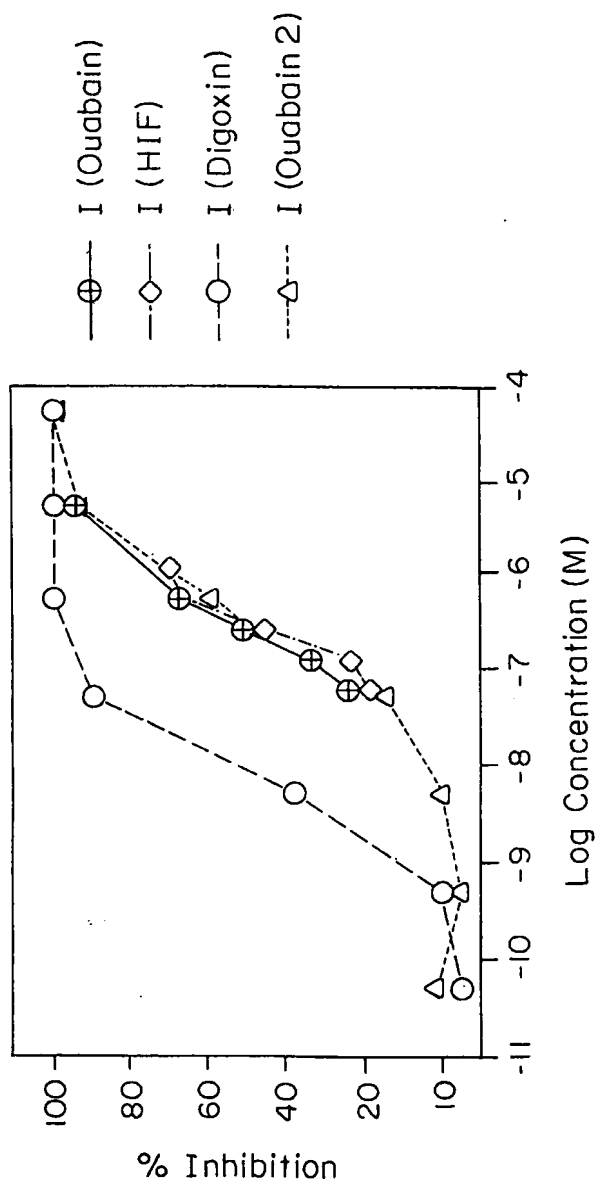


FIG. 1

2/3

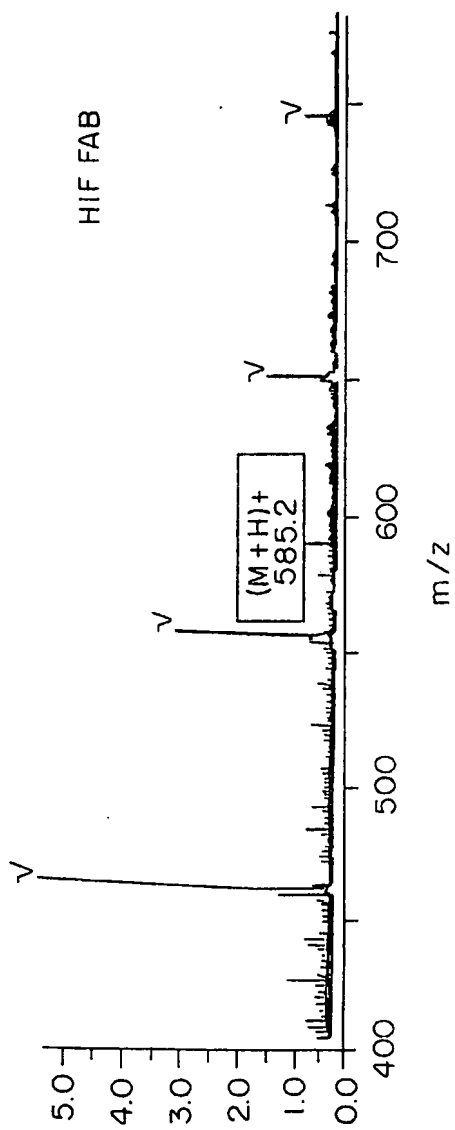
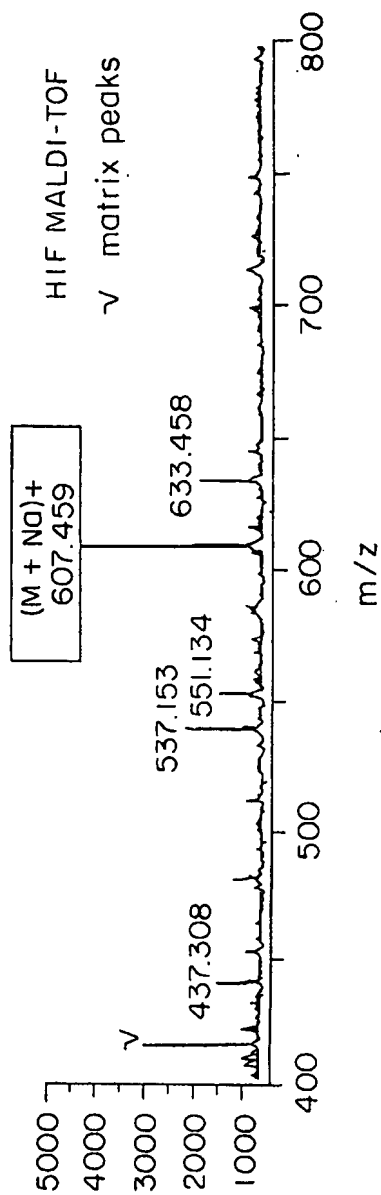


FIG. 2

3/3

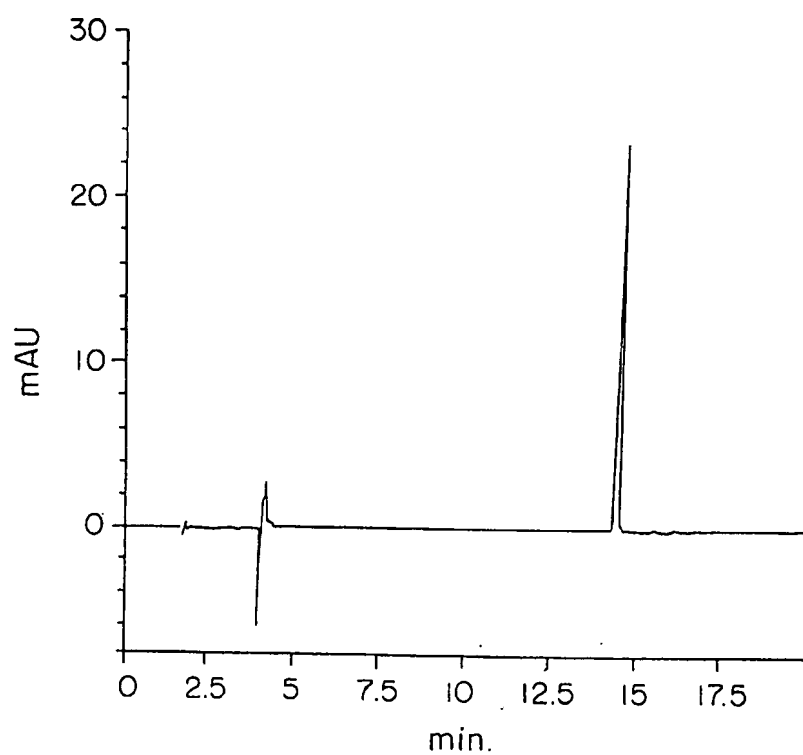


FIG. 3

INTERNATIONAL SEARCH REPORT

National Application No

PCT/US 98/10888

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07H15/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	A. ATYMIK ET AL.: "Physicochemical Characterization of a Ouabain Isomer Isolated from Bovine Hypothalamus." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 90, no. 17, 1 September 1993, pages 8189-8193, XP002074993 WASHINGTON US cited in the application see the whole document -----	1, 4, 13, 21, 29

☐

Further documents are listed in the continuation of box C.

☐

Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

19 August 1998

Date of mailing of the international search report

01/09/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Scott, J